EC-18, A Novel Immune Resolution Accelerator, Improves NASH and Liver Fibrosis

Myung-Hwan Kim[1], Do Young Lee[2], Young-Eun Ko[3], Wonwoo Kim[2], Ji Sun Park[2], Ki-Young Sohn[2], Sun Young Yoon[2] and Jae Wha Kim[3]

[1]Department of Gastroenterology, Asan Medical Center, Asan Medical Center, 88 Olympic-ro 43-gil, Seoul, South Korea, [2]Enzychem Lifesciences Corporation, 10F aT Center 27 Gangnam-daero, Seoul, South Korea, [3]Division of Biomaterials Research, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahak-ro, Deajeon, South Korea

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ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease with significant unmet need. NAFLD encompasses a spectrum of fatty liver disease, ranging from steatosis to nonalcoholic steatohepatitis (NASH) accompanied by hepatocyte ballooning, inflammation and fibrosis. To identify potential new drug for NASH and fibrosis, we investigated whether EC-18 (1-Palmitoyl-2-Linoleoyl-3-Acetyl-rac-Glycerol, PLAG) shows improvement in both NASH and fibrosis via accelerating resolution of inflammation.

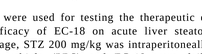
Methods: Total three animal models were used for testing the therapeutic effect of EC-18 against NAFLD. To investigate the efficacy of EC-18 on acute liver steatosis induced by streptozotocin (STZ)-induced β cell damage, STZ 200 mg/kg was intraperitoneally injected to 8-week-old Balb/c mice on Day 1 and vehicle (PBS) and EC-18 were daily administered consecutively from Day 2 to Day 4. To evaluate its efficacy on NASH and fibrosis, we tested EC-18 in a STAM™ mouse model (SMC Lab. Tokyo). Mice were s.c. injected with STZ 200 μg at day after birth and high fat diet for 6 weeks starting at 4 weeks of age. MGL-3196, obeticholic acid (OCA) and EC-18 were orally administered for 3 weeks, starting at 6 weeks of age. To investigate ameliorating effect of EC-18 on inflammation and lipotoxicity in hepatocytes under insulin resistance, we used high-fat-high-fructose (HFHF)-dieted ICR murine modle.

Results: In STZ-induced acute hepatic steatosis model, EC-18 significantly improved STZ-induced histological hepatic steatosis as confirmed by H&E and Oil red O staining. Liver and plasma levels of triglyceride (TG) were decreased following EC-18 treatment (31.3% and 70.7%, respectively, p<0.05). EC-18 restored lipoprotein lipase expression levels in muscle, which were distinctly reduced by STZ treatment, and resulted in the improvement of muscle atrophy and muscle function. In STAM™ NASH and fibrosis model, EC-18 significantly reduced NASH disease activity based on key histological parameters including steatosis & hepatocyte ballooning (p<0.05) compared to the vehicle-treated group. The percentage of fibrosis area (Sirius red-positive area) was significantly decreased in the EC-18 treatment group compared to the vehicle, with mice dosed at the highest concentration (250mg/kg) (p<0.01). Notably, EC-18 reduced liver fibrosis more significantly than OCA and MGL-3196 did. In HFHF-induced insulin resistance mice model, EC-18 attenuated hepatocyte inflammation and cell damage. EC-18 also reduced the secretion of damage-associated molecular patterns (DAMPs), including HMGB1 and lactate dehydrogenase (LDH), from the damaged hepatocytes and attenuated the phosphorylation of RIPK1.

Conclusion: In various mice models, EC-18 ameliorated hepatic steatosis, NASH and liver fibrosis via regulating lipid metabolism in peripheral tissue and attenuating hepatocyte inflammation and cell damage caused by DAMPs. In conclusion, EC-18 could be a promising

EC-18

(**PLAG**: 1-Palmitoyl-2-Linoleoyl-3-Acetyl-rac-Glycerol)



MW 634.99 (small molecule)

- **1-Palmitoyl-2-Linoleoyl-3-Acetyl-rac-Glycerol (PLAG) Rapidly Resolves LPS-Induced Acute Lung Injury Through the Effective Control of Neutrophil Recruitment.** *Front Immunol* **2019, 10:2177**
- **1-Palmitoyl-2-Linoleoyl-3-Acetyl-rac-Glycerol Attenuates Streptozotocin-Induced Pancreatic Beta Cell Damage by Promoting Glucose Transporter 2 Endocytosis.** *Mol Cell Biol* **2019 39(21) e00157**

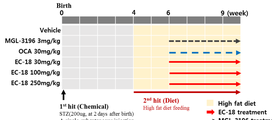
EXPERIMENTAL DESIGN

1. Acute STZ-induced hepatic steatosis mice model

- BALB/c mice (male) aged 8 weeks
- Streptozotocin (STZ): 200mg/kg, IP injection
- Treatment: EC-18 50 or 250mg/kg, p.o.
- Period: 4days

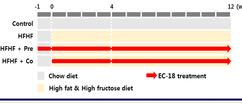
2. STAM™ mice model of NASH (SMC Lab, Japan)

- STAM™ NASH mice model (SMC Lab, Japan)
- C57BL/6J mice
- STZ: 200μg, a single subcutaneous injection
- High fat diet feeding after 4 weeks of age
- Treatment: EC-18 30, 100, 250mg/kg, p.o.; MGL-3196 3mg/kg, p.o.; OCA 30mg/kg, p.o.
- Period: 6-9 weeks



3. High-Fat High-Fructose (HFHF) Diet-fed mice model

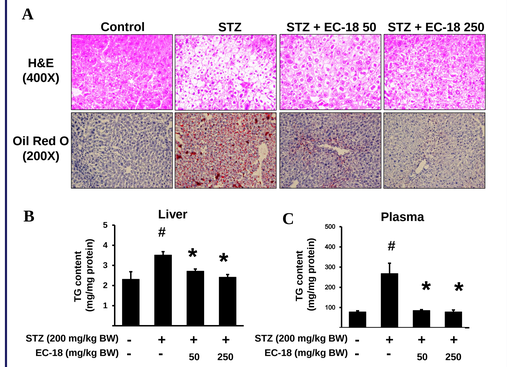
- ICR mice (male) aged 8 weeks
- High Fat, High Fructose diet
- Treatment: EC-18 100mg/kg, p.o. -Pretreated(Pre) or co-treated(Co) EC-18
- Period: 12 weeks



RESULTS

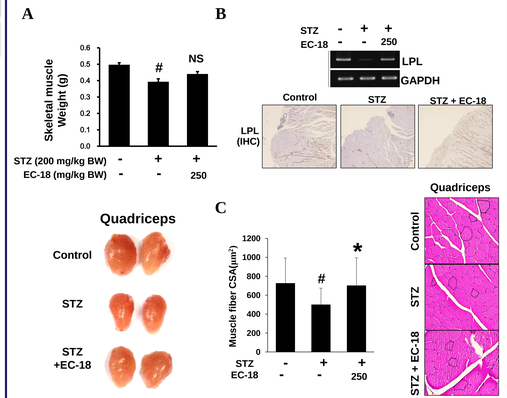
In STZ-induced acute hepatic steatosis mice model

1. EC-18 attenuates hepatic lipid accumulation in STZ-induced hepatic steatosis



(A) H&E and ORO staining in liver tissue. Following sacrifice at the end of the experiment, the liver sections were fixed and then stained with H&E and ORO to examine hepatic lipid deposition. All of the specimens were histologically evaluated under a light microscope (Olympus). Digital images were acquired at a magnification of 200× and 400×. (B&C) TG levels in the liver and plasma. The TG contents of the tissue homogenate and plasma were measured with a commercially available kit (Wako Diagnostics). Quantitative data are expressed as mean±SEM. #p<0.05 compared with the control group. *p<0.05 compared with STZ-induced hepatic steatosis group. H&E, hematoxylin & eosin; ORO, oil red o; TG, triglyceride.

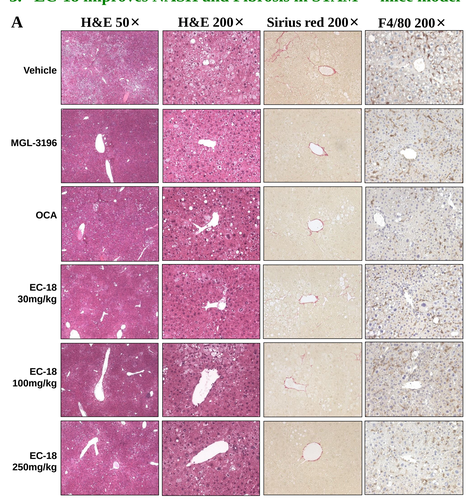
2. EC-18 improves muscle function in STZ-induced hepatic steatosis

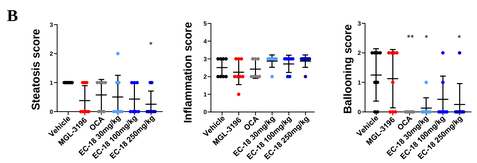


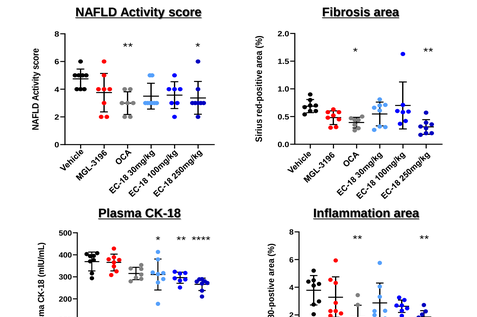
(A) Muscle weight and its gross morphology from mice. Muscle was collected at the end of the experiment, and fresh weights were recorded. (B) Relative mRNA expression and immunohistochemical image of LPL in muscle tissue. (C) The CSA of myofibers and H&E staining in quadriceps. Quantitative data are expressed as mean±SEM. #p<0.05 compared with control. *p<0.05 compared with STZ-induced hepatic steatosis group. LPL, lipoprotein lipase; CSA, cross-sectional area.

In STAM™ model of NASH (SMC Lab. Japan)

3. EC-18 improves NASH and Fibrosis in STAM™ mice model



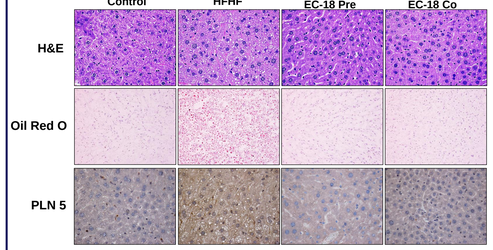




Reduction of NAS score and fibrosis in STAM™ mice following the EC-18 treatment. (A) Representative H&E (50× and 200×)-, Sirius red (200×)-, and F4/80 (200×)-stained liver sections from vehicle, MGL-3196, OCA, and EC-18-treated STAM™ mice collected at week 9, showing the histological features for the different groups. (B) Comparison of NAS score and fibrosis areas from mouse liver specimens in 9-week-old mice from each treatment group. Individual components of the NAS, including steatosis, lobular inflammation, and hepatocellular ballooning scores, are shown for each cohorts. Vehicle, n = 8; MGL-3196, n = 8; OCA, n = 7; EC-18 30mg/kg, n = 8; EC-18 100mg/kg, n = 7; EC-18 250mg/kg, n =8. *p <0.05, **p<0.01, ****p<0.0001 vs Vehicle Control. For NAS, statistical analyses were performed using Dunnett's Multiple Comparison Test. For other data, statistical analyses were performed using Bonferroni Multiple Comparison Test.

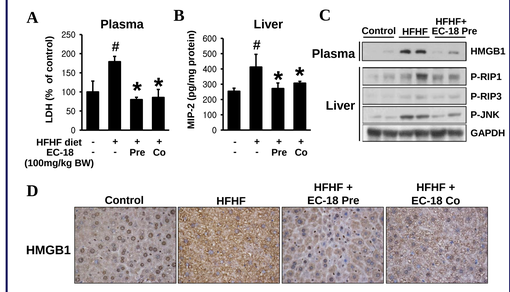
In HFHF Diet-fed mice model

4. EC-18 prevents hepatic steatosis in HFHF diet-fed mouse model



Histological analysis in liver tissue. Following sacrifice at the end of the experiment, the liver sections were fixed and then stained with H&E and ORO to examine hepatic lipid deposition. A lipid droplet protein, PLN5 was analyzed by immunohistochemistry. All of the specimens were histologically evaluated under a light microscope (Olympus). Digital images were acquired at a magnification of 200 and 400×. H&E, hematoxylin & eosin; ORO, oil red o; PLN5, perilipin5.

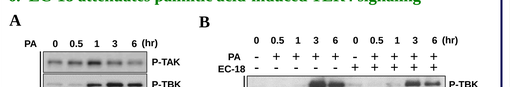
5. EC-18 prevents hepatic inflammation and damage in HFHF diet-fed mouse model



(A) LDH level in blood. At the end of experiment, the amount of LDH in plasma was determined with commercially available kits (Thermo Fisher Scientific) according to the manufacturer's instructions. (B) MIP-2 secretion in liver homogenate measured by ELISA. (C) HMGB1 expression in plasma, phosphorylation of RIPK1/3, and JNK in liver tissue. To analyze the released HMGB1, plasma sample and tissue lysate were separated by SDS-PAGE and transferred. The protein extracts were immunoblotted with HMGB1, p-RIP1/3, p-JNK and GAPDH antibody and detection was conducted using Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation). (D) HMGB1 in liver tissue was analyzed by immunohistochemistry and presented at 200X magnification. Quantitative data are expressed as mean± SD. #p<0.05 compared with control. *p<0.05 compared with HFHF-diet-fed mice group. LDH, lactate dehydrogenase; MIP-2, macrophage inflammatory protein 2; HMGB1, high mobility group box 1; p-RIP1/3, phospho-receptor interacting serine/threonine kinase 1/3; p-JNK, phospho-c-Jun N-terminal kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

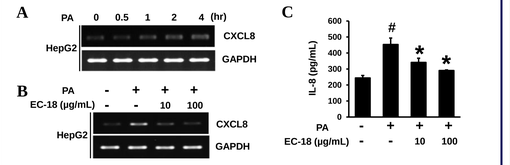
In Vitro study

6. EC-18 attenuates palmitic acid-induced TLR4 signaling



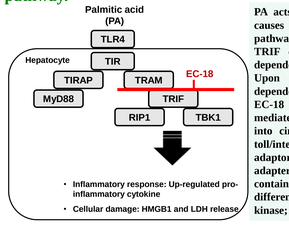
(A) HepG2 cells were treated with 400 μM palmitic acid (PA) for indicated time periods. (B) HepG2 cells were pre-treated with 100 μg/mL EC-18 for 2hr, and then stimulated with 400 μM PA. TLR4 signaling-associated protein expression determined by western blot. TLR4, Toll-like receptor 4; P-TAK, phospho-transforming growth factor-β-activated kinase; p-TBK, phospho-TANK-binding kinase; P-RIP, phospho-receptor-interacting protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

7. EC-18 attenuates CXCL8 expression induced by palmitic acid



(A) HepG2 cells were treated with 400 μM palmitic acid (PA) at the indicated time periods. (B&C) HepG2 cells were pre-treated with 10 and 100 μg/mL EC-18 for 2hr, and then stimulated with 400 μM PA. After 4h and 24h, cells and supernatant were harvested respectively. (B) CXCL8 mRNA expression determined by RT-PCR. (C) Secreted IL-8 was determined by ELISA. Quantitative data are expressed as mean± SD. #p<0.05 compared with control. *p<0.05 compared with PA-exposed group. CXCL8, C-X-C motif chemokine ligand 8; IL-8, interleukin 8.

8. Schematic illustration of the role of EC-18 on TLR4 signaling pathway.



PA acts as a metabolic ligand to TLR4. TLR4 causes two distinctive downstream signaling pathways, including TIRAP-MyD88 and TRAM-TRIF dependent pathways. PA induces TRIF dependent RIP1 and TBK1 phosphorylation. Upon EC-18 treatment, it regulates TRIF dependent RIP1 and TBK1 activation. Therefore, EC-18 attenuates the TLR4/TRIF signaling-mediated CXCL8 production and HMGB1 release into circulation. TLR4, toll-like receptor; TIR, toll/interleukin-1 receptor; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adapter-inducing IFN-β; TIRAP, TIR domain-containing adapter protein; MyD88, myeloid differentiation factor 88; TBK, TANK-binding kinase; RIP1, receptor-interacting protein 1.

CONCLUSION

- **In various NAFLD mice models, EC-18, as an immune resolution accelerator, ameliorated hepatic steatosis, NASH, and liver fibrosis via enhancing lipid metabolism in peripheral tissue.**
- **EC-18 significantly reduced NFALD activity score based on key histological parameters including steatosis and hepatocyte ballooning.**
- **EC-18 significantly reduced liver fibrosis, plasma CK-18 fragments, and inflammation area assessed compared with vehicle control and reference compounds.**
- **EC-18 attenuated hepatocyte inflammation and cell damage caused by damage associated molecular patterns (DAMPs).**
- **EC-18 was superior to reference compounds (OCA, MGL3196, and CVC), currently being tested in phase 3 clinical trial, in terms of mitigating NASH symptoms and reducing the progression to fibrosis.**
- **Based on these results, EC-18 could be a promising therapeutic candidate to resolve NASH and to prevent progression to liver fibrosis.**